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Original Article

Mutations in the *NOG* gene are commonly found in congenital stapes ankylosis with symphalangism, but not in otosclerosis

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Human noggin (*NOG*) is a responsible gene for multiple synostosis syndrome (SYNS1) and proximal symphalangism (SYM1), two conditions that are recently known to be within a wider range of clinical manifestations of stapes ankylosis with symphalangism. This study was performed to determine the range of phenotype caused by *NOG* mutations, using Japanese patients with various phenotypes including sporadic inherited SYM1, dominantly inherited SYM1, stapes ankylosis with broad thumb and toes (Teunissen and Cremer syndrome). In addition, 33 patients with typical otosclerosis (without symphalangism) were studied. Direct sequencing analysis disclosed three novel mutations of the *NOG* gene in three SYM1 families. None of the otosclerosis patients without symphalangism had *NOG* mutations, indicating that *NOG* mutations may be restrictively found within patients with various skeletal abnormalities. These results together with the literature review indicated that there are no clear genotype–phenotype correlations for NOG mutations. With regard to surgical outcome, most of the patients in these three families with *NOG* mutations showed remarkable air–bone gap recovery after stapes surgery. Molecular genetic testing is useful to differentiate syndromic stapes ankylosis from otosclerosis, and even mild skeletal anomalies can be a diagnostic indicator of NOG-associated disease.

Conflict of interest

The authors declare no conflict of interest.

S Usami[a], S Abe[a,b], S Nishio[a], Y Sakurai[c], H Kojima[c], T Tono[d] and N Suzuki[a]

[a]Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan, [b]Department of Otorhinolaryngology, Abe ENT Clinic, Ota-ku, Japan, [c]Department of Otorhinolaryngology, Jikei University School of Medicine, Minato-ku, Tokyo, Japan, and [d]Department of Otorhinolaryngology, University of Miyazaki Faculty of Medicine, Miyazaki, Japan

Key words: – otosclerosis – stapes ankylosis – stapes ankylosis with broad – SYM1 – symphalangism – Teunissen and Cremer syndrome – thumb and toes

Corresponding author: Shin-ichi Usami, Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan.
Tel.: +81 263 37 2666;
fax: +81 263 36 9164;
e-mail: usami@shinshu-u.ac.jp

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Human noggin (*NOG*) is a responsible gene for a wide range of clinical manifestations of stapes ankylosis with symphalangism. Proximal symphalangism (SYM1: MIM #185800) (1) is known as an autosomal dominant disorder with high penetrance. The most common clinical features are the immobility of the proximal interphalangia (PIP) joints of the hands and toes, and congenital conductive hearing loss due to stapes ankylosis. Multiple synostosis syndrome (SYNS1: MIM#186500) (1) is characterized by a more severe phenotype of the proximal symphalangism, such as progressive and expanded bony fusion of joints and unique facial manifestations. In addition, mutations in NOG have been identified in Tarsal–Carpal Coalition syndrome (TCC: MIM#186570) (2), stapes ankylosis with broad thumb and toes (MIM#184460) (Teunissen and Cremer syndrome) (3), and Brachydactyly type B2 (BDB2: MIM#611377) (4).

Otosclerosis (MIM#166800) is known as the single most common cause of progressive conductive hearing loss, characterized by abnormal bone remodeling in the otic capsule. Although there are a small number of familial cases that are likely to be monogenic, the majority of cases are sporadic. A series of studies has suggested that this condition involves both genetic and environmental factors (5).

Typical otosclerosis was included in this study because it is an interesting question as to whether some of the typical otosclerosis is a continuum of a category of disease caused by *NOG* mutations. We thought this may be true because (i) within SYM1, *NOG* mutations were found in patients with minor skeletal anomalies without symphalangism (3), and (ii) stapes ankylosis is an important phenotype of the animal model for $NOG^{+/-}$ mice (6).

To date, no detailed survey was available for *NOG* mutations in the stapes ankylosis patients with symphalangism in Asian populations. Therefore this study was undertaken to address whether *NOG* mutations are also causative and commonly found in those populations and if so, whether there is a different mutation spectrum.

In addition, previously reported *NOG* mutations were reviewed to determine their spectrum as well as whether there are any particular genotype–phenotype correlations caused by *NOG* mutations.

Materials and methods

Subjects

We ascertained three Japanese families to be associated with conductive hearing loss and symphalangism, including an autosomal dominant SYM1 family, a sporadic SYM1 case with normal parents, and an autosomal dominant stapes ankylosis with broad thumb and toes (Teunissen and Cremer syndrome) family. Thirty-three Japanese otosclerosis patients, who underwent stapes surgery, were also screened for mutations in the *NOG* gene. Their clinical symptoms, including ages at surgery (36–77 years old: average 54.4 years old), onset age (15–57 years old: average 37.3 years old), gender (10 male and 23 female), laterality (9 unilateral and 24 bilateral), and hearing threshold (average 63.1 dB), are summarized in Table 1. Average onset age, was hearing threshold, was evaluated using pure-tone audiometry classified by a pure-tone average over 250, 500, 1000, 2000, and 4000 Hz. By detailed anamnestic and medical examination, no patients had any associated skeletal abnormalities. All of the patients were sporadic cases and no similar condition was observed within their familial members. Satisfactory outcomes after stapes surgery were obtained in all 33 subjects.

We obtained informed consent for participation in this project from each subject and also from 192 normal control subjects who were unrelated Japanese individuals without any noticeable hearing loss evaluated by auditory testing. Otologic examination, audiometric analysis, and radiologic imaging were carried out for each patient.

Family 1

As shown in the pedigree (Fig. S1a), patient #991 was diagnosed with symmetric conductive hearing loss of 50 dB (Fig. 1b) at the age of 6 years. Tympanography indicated type A sclerosis and absence of the stapedius reflex, whereas otomicroscopy results were normal. Temporal bone computed tomography (CT) scan revealed no inner or middle ear malformations. Her hearing level was stable and non-progressive, and she received hearing aids in both ears. At the age of 17, exploratory tympanotomy of the left ear showed bony fixation of the footplate without any other deformities in the middle ear and stapedotomy using a Teflon piston and wire was performed, resulting in a remarkable improvement in hearing. One year later, stapedotomy was undertaken in her right ear also. After the surgery, the postoperative hearing levels showed 20–30 dB and she did not use her hearing aids. The X-ray presented in Fig. 1 shows symphalangism in the PIP joints of the second to fifth fingers of both hands and in the distal interphalangeal (DIP) joints of the left second and fifth fingers and of the right fifth finger. There was symphalangism in both hands, resulting in limited mobility of the fingers. Symphalangism (fixation of the proximal interphalangeal joint) in both feet was also found. The ankylosis was confirmed by X-ray examination

Table 1. Clinical symptoms of Otosclerosis patients

Patient number	Age	Gender	Onset age	Affected side	Hearing threshold (right)	Hearing threshold (left)
1	48	M	36	Bilateral	68.3	56.3
2	59	F	45	Bilateral	56.3	56.3
3	43	F	38	Left	10.8	64.0
4	36	M	25	Bilateral	61.3	66.3
5	46	F	40	Right	38.0	19.0
6	44	F	33	Bilateral	59.0	66.0
7	65	F	49	Bilateral	81.0	63.0
8	77	F	57	Bilateral	104.0	105.0
9	45	F	41	Bilateral	56.0	69.0
10	44	F	30	Left	30.0	80.0
11	61	F	44	Bilateral	23.0	68.0
12	58	F	49	Right	76.0	35.0
13	43	M	25	Left	14.0	49.0
14	58	F	47	Bilateral	53.0	50.0
15	54	F	38	Bilateral	57.0	45.0
16	53	F	40	Right	58.0	6.0
17	44	F	25	Bilateral	53.0	56.0
18	62	F	48	Bilateral	42.5	52.5
19	43	F	33	Bilateral	27.0	45.0
20	57	F	40	Left	26.0	65.0
21	65	F	15	Bilateral	53.0	50.0
22	54	M	46	Bilateral	50.0	30.0
23	48	F	23	Bilateral	71.0	56.0
24	62	F	39	Bilateral	38.0	37.0
25	76	F	43	Bilateral	78.0	75.0
26	71	M	41	Bilateral	91.3	97.5
27	71	F	40	Bilateral	126.3	110.0
28	45	M	45	Left	42.5	73.75
29	41	F	30	Bilateral	98.8	95
30	44	M	30	Left	30.0	76.3
31	44	M	30	Right	58.8	38.8
32	64	M	35	Bilateral	51.0	46.0
33	70	M	30	Bilateral	48.8	53.8

F, female; M, male.

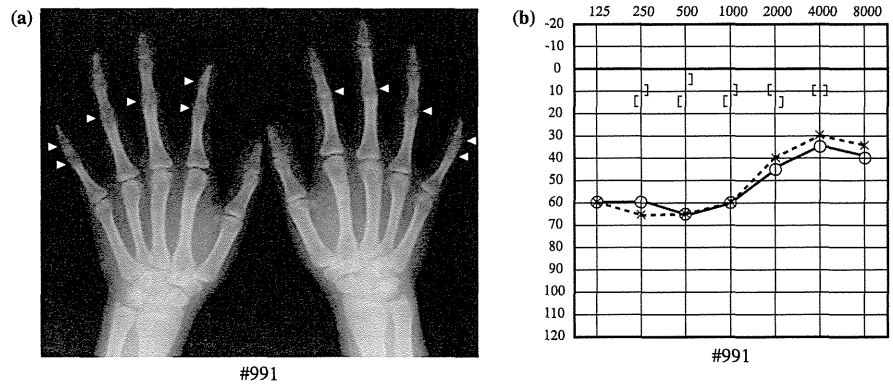

Fig. 1. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patient #991. (**b**) Audiograms from patient #991 showing conductive hearing loss.

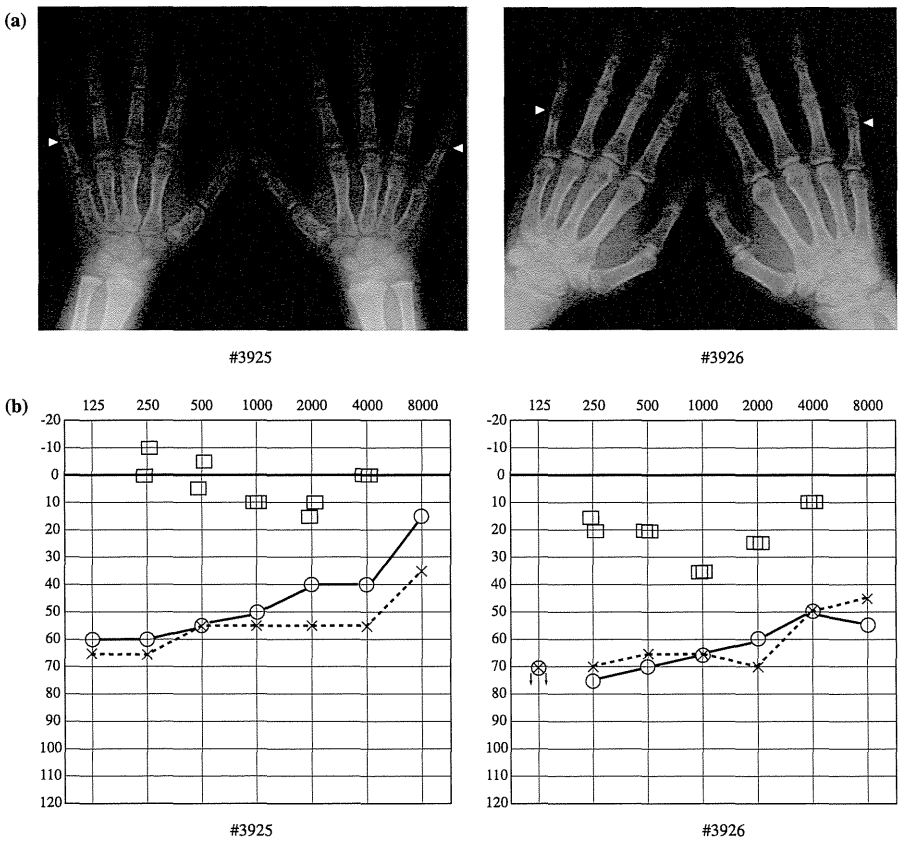

Fig. 2. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patients #3925 and #3926. (**b**) Audiograms from patients #3925 and #3926 showing conductive hearing loss.

(Fig. 1a). Congenital hyperopia (only in this patient within the family) was also present.

Family 2

As shown in the pedigree (Fig. S2a), a girl and her father (patients #3925 and #3926) visited our hospital due to bilateral hearing loss. Audiograms indicated bilateral mixed hearing loss (Fig. 2b). Anamnestic evaluation suggested that the hearing loss was non-progressive without any associated symptoms such as ear fullness, tinnitus or vertigo. Patient #3926 underwent stapedotomy at the age of 42, achieving significant recovery of his hearing. There was symphalangism in the PIP joint of both fifth fingers, resulting in limited mobility of the fingers. Fixation of the proximal interphalangeal joint was not found in either foot. The ankylosis was confirmed by X-ray examination

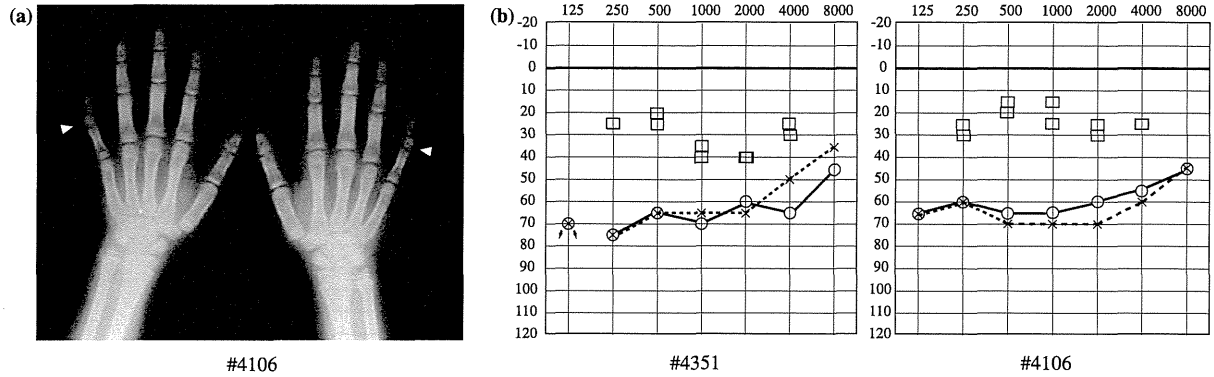

Fig. 3. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patients #4106. (**b**) Audiograms from patients #4106 and #4351 showing conductive hearing loss.

(Fig. 2a). Congenital hyperopia was not present in this family.

Family 3

The pedigree shows hearing loss was inherited in four generations, indicating autosomal dominant inheritance (Fig. S3a). In addition to conductive hearing loss (Fig. 3b), the family members were associated with the following clinical phenotype in various degrees: hyperopia, broad thumbs and first toes, symphalangism, syndactyly, and fused cervical vertebrae (Fig. S3a). The clinical diagnosis was therefore stapes ankylosis with broad thumb and toes (3) or Teunissen and Cremer syndrome (7). Patient #4351 had conductive hearing loss, hyperopia, broad thumbs and first toes, symphalangism, and syndactyly. She noted her hearing loss around age 10. Stapedotomy was performed when she was 37 (right) and 38 (left) years old, achieving significant recovery of hearing. Patient #4106 had conductive hearing loss, hyperopia, broad thumbs and first toes, and fused cervical vertebrae. His hearing loss was noted around age 3 and was diagnosed at the age of 8. Stapedotomy was performed when he was 9 (right) and 10 (left) years old, achieving significant recovery of hearing.

Mutation identification

Human *NOG* gene coding is constituted of one single exon, in which an open reading frame of 696 nucleotides encodes a NOG polypeptide of 232 amino acids. A sequence obtained from GeneBank U31202 was used to design primers containing the entire coding region of *NOG*. Two fragments to entirely cover the coding region of NOG were amplified with polymerase chain reaction (PCR) and two specific primer pairs, as follows: F1, 5′-CTTGTGTGCCTTTCTTCCGC-3′; R1, 5′-TACTGGATGGGAATCCAGCC-3′; and F2, 5′-TACGACCCAGGCTTCATGGC-3′; R2, 5′-TAGCACG AGCACTTGCACTC-3′.

PCR reactions were carried out in 25 μl total volume containing 40 ng of genomic DNA, 10 pmol of each primer, 2 mM dNTPs, ×10 PCR buffer and 0.2 U of ExTaq polymerase (Takara, Tokyo, Japan). PCR conditions were denaturing at 94°C for 2 min; 35 cycles at 96°C for 30 s, 60°C for 30 s, 72°C for 1 min extension, with a final extension step at 72°C for 5 min in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer, Foster City, CA). PCR products were purified with a Suprec filter (Takara) and sequenced directly, using four primers (F1, R1, F2, and R2) and ABI BigDye terminators, on an ABI 3100 sequencer (Perkin-Elmer).

Results

Three novel mutations of the *NOG* gene were found by direct sequencing analysis in three families, whose common clinical features were compatible with SYM1, i.e. immobility of the PIP joints of the hands and toes, and congenital conductive hearing loss due to stapes ankylosis. Patient #991 of family 1 had a heterozygous G>T transversion at nucleotide 551 (Fig. S1b), predicting a cysteine (C) for phenylalanine (F) substitution at amino acid 184 (C184F) in the coding region of NOG. Since the C184F mutation was not found in either parent and was found only in the proband (patient #991), it was suggested that the mutation arose *de novo* in only the affected individual. Patients #3925 and #3926 of family 2 had a heterozygous T>A transversion at nucleotide 463 (Fig. S2b), predicting a cysteine (C) for serine (S) substitution at amino acid 155 (C155F) in the coding region of NOG. Patients #4106 and #4351 had a heterozygous C215X mutation.

None of the otosclerosis patients had *NOG* mutations. These three mutations were not observed in any of the other family members nor in the 192 unrelated Japanese controls (384 chromosomes).

Discussion

This study identified three novel mutations in the *NOG* gene in families with symphalangism, being consistent with the previous work showing that *NOG* is the responsible gene for SYM1 and stapes ankylosis with

Table 2. NOG mutations reported in SYM1, SYNS1, TCC, BDB2 and TCS families

Nucleotide change	Amino acid	Family information	Phenotype	Evolutionary conservation +	Domain/structure/motif ++	Authors
c. 58delC	Frameshift	Japanese, AD	SYNS1	—	—	Takahashi (8)
c. 103C>G	p. P35A	German, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 103C>T	p. P35S	Turkish, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 103C>T	p. P35S	Israeli, AD	SABTT	Conserved	Finger/clip region Interface of NOG and BMP7	Hirshoren (9)
c. 103C>T	p. P35S	Italian, AD	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Mangino (10)
c. 104C>G	p. P35R	NI, sporadic	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Gong (1)
c. 104C>G	p. P35R	NI, AD	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Dixon (2)
c. 106G>C	p. A36P	Danish, AD	BDB2	Almost conserved[a]	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 110C>G	p. P37R	Belgian, AD	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (11)
c. 124C>G	p. P42A	Belgian, *de novo*	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (12)
c. 125C>G	p. P42R	NI, AD	SYNS1	Conserved	Finger/clip region Interface of NOG and BMP7	Oxley (13)
c. 129-130dup	Frameshift	Dutch, AD	SABTT	—	—	Weekamp (14)
c. 142G>A	p. E48K	Japanese, sporadic	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Kosaki (15)
c. 142G>A	p. E48K	Iranian, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 149C>G	p. P50R	Belgian, *de novo*	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (12)
c. 252-253 insC	Frameshift	NI, AD	SABTT	—	—	Brown (3)
c. 304delG	Frameshift	Dutch	SYM1	—	—	Thomeer (16)
c. 328C>T	p. Q110X	Italian, AD	SABTT	Conserved	—	Brown (3)
c. 386T>A	p. L129X	Japanese, AD	SYM1	Almost conserved[b]	—	Takahashi (8)
c. C391C>T	p. Q131X	Dutch	SYM1	Almost conserved[b]	—	Thomeer (16)
c. 463T>A	p. C155S	Japanese, AD	SYM1	Conserved	Conserved cysteine of cysteine knot I	Present study
c. 499C>G	p. R167G	North American, sporadic	BDB2	Conserved	—	Lehmann (4)
c. 551G>A	p. C184Y	Japanese, sporadic	SYM1	Conserved	Conserved cysteine of cysteine knot III	Takahashi (8)
c. 551G>T	p. C184F	Japanese, sporadic	SYM1	Conserved	Conserved cysteine of cysteine knot III	Present study
c. 559C>T	p. P187S	British, AD	BDB2	Conserved	—	Lehmann (4)
c. 561del	Frameshift	Dutch, AD	SABTT	—	—	Weekamp (14)
c. 565G>T	p. G189C	Dutch, AD	SYM1	Conserved	—	Gong (1)
c. 568A>G	p. M190V	NI, AD	SYNS1	Conserved	—	Oxley (13)
c. 608T>C	p. L203P	Dutch, AD	SABTT	Conserved	β-Sheet 3 of NOG structure	Weekamp (14)
c. 611G>T	p. R204L	NI, AD	TCC	Conserved	β-Sheet 3 of NOG structure	Dixon (2)
c. 614G>A	p. W205X	sporadic	SYNS1	Conserved	—	Dawson (17)
c. 615G>C	p. W205C	Belgian, AD	SYNS1	Conserved	β-Sheet 4 of NOG structure	Declau (18)
c. 615G>C	p. W205C	American, sporadic	SABTT	Conserved	β-Sheet 4 of NOG structure	Emery (19)
c. 645C>A	p. C215X	Japanese, AD	SABTT	Conserved	Disulphide bounds in cysteine knot motif to stabilize finger 2 structure	Present study
c. 649T>G	p. W217G	Hawaiian, AD	SYNS1	Conserved	β sheet 4 of NOG structure	Gong (1)
c. 659-660TC>AT	p. I220N	Belgian, AD	SYM1	Almost conserved[a]	Interaction region to BMP-type binding epitope	Gong (1)
c. 659T>A	p. I220N	NI, AD	SYM1	Almost conserved[a]	Interaction region to BMP-type binding epitope	Gong (1)
c. 664T>G	p. Y222D	Belgian, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 665A>G	p. Y222C	American, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 665A>G	p. Y222C	NI, AD	TCC	Conserved	Interaction region to BMP-type binding epitope	Dixon (2)
c. 668C>T	p. P223L	NI, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 696C>G	p. C232W	Germany, AD	SYM1	Conserved	Intermolecular disulphide bounds to stabilize NOG dimmer structure	Rudnik-Schöneborn (20)
17q22 long deletion		Japanese, sporadic	SYNS1	—	—	Shimizu (21)

+, evolutionary conservation was evaluated by the NCBI data base; ++, the domain/structure/motif are based on a hypothesized protein structure; AD, autosomal dominant; BDB2, brachydactyly type B2; BMP, bone morphogenetic protein; FOP, fibrodysplasia ossificans progressiva; NI, no information; NOG, noggin; SABTT, stapes ankylosis with broad thumbs and toes; SYNS1, multiple synostosis syndrome; SYM1, proximal symphalangism; TCC, trasal–carpal coalition syndrome.

[a]Residue is conserved across mammalians except for zebrafish.

[b]Residue is conserved across mammalians except for zebrafish and chicken.

broad thumb and toes/Teunissen and Cremer syndrome. One mutation was a nonsense mutation (C215X), leading to a truncated protein, and was likely to be a pathologic mutation. The other two mutations are also likely to be pathologic rather than functionally neutral polymorphic changes because: (i) none were found in any of the controls, (ii) the alignment of NOG sequences from human, mouse, chicken, *Xenopus laevis* and zebrafish showed that C155 and C184 are well-conserved amino acids in all species (data not shown), and (iii) all affected subjects showed similar phenotypes.

To date, 36 *NOG* mutations have been reported in SYM1, SYNS1, TCC, BDB2 and TCS families (Table 2). Although the *NOG* mutations have been reported mainly in dominant families (Table 2), *de novo NOG* mutations have also been reported in sporadic SYM1 (8) and sporadic SYNS1 (1). Therefore, genetic investigation may be needed for determining pathogenesis of congenital stapes ankylosis with stiffness of the PIP joints, even in sporadic cases. A milder phenotype (3) as well as the present case with minor joint anomalies in family 2 indicated that it may be clinically important to check such skeletal abnormalities when diagnosing and treating patients with stapes ankylosis, because it may be difficult to differentiate congenital stapes ankylosis from otosclerosis when conductive hearing loss is delayed to adulthood.

Whether *NOG* mutations can be found more frequently in sporadic conductive hearing loss patients is an interesting question. In this study, mutations were not found in any otosclerosis patients who did not have any associated abnormality. Therefore, typical otosclerosis is not a continuum of the category of diseases associated with *NOG* mutations. These results, together with the previous literature, indicate that the *NOG* mutations are restrictively found within patients with various skeletal abnormalities regardless of severity. It is noted that the reported *NOG* mutation in mild cases (patients with stapes ankylosis without symphalangism) have minor skeletal abnormalities such as broad thumbs and great toes (3), but these cases had symphalangism in the little fingers only.

A review of the reported 41 mutations showed that, interestingly, the majority of *NOG* mutations are located in the evolutionally well conserved and therefore functionally critical region (Table 2), suggesting that this region might be functionally relevant in NOG polypeptides. This study added three novel *NOG* mutations in conserved cysteine residue within the cystine knot motif and confirmed that *NOG* is a causative gene for this category of disease. In addition, there was no particular racial-specific founder mutation within this gene (Table 2). With regard to a genotype–phenotype correlation, phenotypes seem to be independent of the location of the mutation and type of mutations (Table 2). Other genetic factors and/or interacted proteins may also be involved in determining clinical phenotypes.

With regard to surgical outcome, stapes surgery for conductive hearing loss due to *NOG* mutations may be a good therapeutic option for most cases. In fact, two of these seven patients who underwent stapes surgery (#991 and #4106) had hearing deterioration 3–10 years after the initial surgery, in accordance with a previous report (3, 22), hypothesizing that excessive bony overgrowth and refixation of the ossicle chain may occur after initially successful surgery. The other cases in this study maintained good hearing even after long-term follow-up periods (more than 10 years). Therefore, surgical outcome should be carefully evaluated after long-term observation. Careful explanation of possible limitations of surgical treatment and alternative treatment options such as a bone-anchored hearing aid may be appropriate for such patients with this genetic background.

The identification of the causative genes responsible for various middle/inner ear diseases will enable us to classify new congenital deafness groups in the future, and lead to clinical application in the diagnosis of middle ear disorders and better counseling for the selection of ideal intervention.

Supporting Information

The following Supporting information is available for this article:

Fig. S1. (**a**) Pedigree of family 1. Filled symbol represents the affected individual. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a G to T change at nucleotide 551 in patient #991. This substitution causes codon 184 to change from TGC (cysteine: C) to TTC (phenylalanine: F).

Fig. S2. (**a**) Pedigree of family 2. Filled symbols represent the affected individuals. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a T to A change at nucleotide 463 in patients #3925 and #3926. This substitution causes codon 155 to change from TGC (cysteine: C) to AGC (serine: S).

Fig. S3. (**a**) Pedigree of family 3. Filled symbols represent affected symptoms (conductive hearing impairment, hyperopia, and finger malformation. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a C to A change at nucleotide 645 in patients #4106 and #4351. These two patients had a heterozygous C215X mutation. This nonsense mutation (C215X) leads to a truncated protein.

Additional Supporting information may be found in the online version of this article.

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Original Article

A prevalent founder mutation and genotype–phenotype correlations of *OTOF* in Japanese patients with auditory neuropathy

T Matsunaga[a], H Mutai[a], S Kunishima[b], K Namba[a], N Morimoto[c], Y Shinjo[d], Y Arimoto[e], Y Kataoka[f], T Shintani[g], N Morita[h], T Sugiuchi[i], S Masuda[j], A Nakano[e], H Taiji[c] and K Kaga[d]

[a]Laboratory of Auditory Disorders, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan, [b]Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan, [c]Department of Otorhinolaryngology, National Center for Child Health and Development, Tokyo, Japan, [d]National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan, [e]Division of Otorhinolaryngology, Chiba Children's Hospital, Chiba, Japan, [f]Department of Otolaryngology, Head and Neck Surgery, Okayama University Postgraduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Japan, [g]Department of Otolaryngology, Sapporo Medical University School of Medicine, Sapporo, Japan, [h]Department of Otolaryngology, Teikyo University School of Medicine, Tokyo, Japan, [i]Department of Otolaryngology, Kanto Rosai Hospital, Kawasaki, Japan, and [j]Department of Otorhinolaryngology, Institute for Clinical Research, National Mie Hospital, Tsu, Japan

Key words: auditory neuropathy – genotype–phenotype correlation – mutation – non-syndromic hearing loss – *OTOF*

Corresponding author: Tatsuo Matsunaga MD, PhD, Laboratory of Auditory Disorders and Department of Otolaryngology, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro, Tokyo 152-8902, Japan.

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Auditory neuropathy is a hearing disorder characterized by normal outer hair cell function and abnormal neural conduction of the auditory pathway. Aetiology and clinical presentation of congenital or early-onset auditory neuropathy are heterogeneous, and their correlations are not well understood. Genetic backgrounds and associated phenotypes of congenital or early-onset auditory neuropathy were investigated by systematically screening a cohort of 23 patients from unrelated Japanese families. Of the 23 patients, 13 (56.5%) had biallelic mutations in *OTOF*, whereas little or no association was detected with *GJB2* or *PJVK*, respectively. Nine different mutations of *OTOF* were detected, and seven of them were novel. p.R1939Q, which was previously reported in one family in the United States, was found in 13 of the 23 patients (56.5%), and a founder effect was determined for this mutation. p.R1939Q homozygotes and compound heterozygotes of p.R1939Q and truncating mutations or a putative splice site mutation presented with stable, and severe-to-profound hearing loss with a flat or gently sloping audiogram, whereas patients who had non-truncating mutations except for p.R1939Q presented with moderate hearing loss with a steeply sloping, gently sloping or flat audiogram, or temperature-sensitive auditory neuropathy. These results support the clinical significance of comprehensive mutation screening for auditory neuropathy.

Conflict of interest

The authors declare no conflict of interest.

Tel.: +81 3 3411 0111;
fax: +81 3 3412 9811;
e-mail:
matsunagatatsuo@kankakuki.go.jp

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Auditory neuropathy (AN) is a hearing disorder characterized by normal outer hair cell function, as revealed by the presence of otoacoustic emissions (OAE) or cochlear microphonics, and abnormal neural conduction of the auditory pathway, as revealed by the absence or severe abnormality of auditory brainstem responses (ABR) (1). Hearing disorders having the same characteristics have also been reported as auditory nerve disease in adult cases (2). Individuals with AN invariably have difficulties in understanding speech (3), and approximately 10% of infants diagnosed with profound hearing loss have AN (3, 4).

About 50% of subjects with congenital or early-onset AN have risk factors such as perinatal hypoxia, whereas the remaining 50% of subjects are likely to have a genetic factor (3, 5). To date, four loci responsible for non-syndromic AN have been mapped: DFNB9 caused by *OTOF* mutation and DFNB59 caused by *PJVK* mutation, both of which are responsible for autosomal recessive AN; AUNA1 caused by DIAPH3 mutation, which is responsible for autosomal dominant AN; and AUNX1, which is responsible for X-linked AN (6–9). Mutations in *OTOF*, which contains 50 exons and encodes short and long isoforms of otoferlin (10), are the most frequent mutations associated with AN with various frequency depending on the population studied (11–15). Most *OTOF* genotypes have been associated with stable, severe-to-profound hearing loss with only a few exceptions (11–20). Studies of genetic backgrounds and clinical phenotypes in various populations will extend our knowledge of genotype–phenotype correlations and may help in the management and treatment of AN.

Materials and methods

Subjects

We enrolled 23 index patients of unrelated Japanese families with congenital or early-onset AN. Diagnosis of hearing loss was made by age 2 in all patients except for one, who had mild hearing loss diagnosed at age 9. All patients had non-syndromic AN in both ears, and they were collected from all over Japan as part of a multicentre study of AN. Patients with hearing loss of possible environmental risk factors for AN such as neonatal hypoxia or jaundice were excluded. With regard to the family history, one patient had a brother having congenital AN and all others were simplex. DNA samples and medical information were obtained from each proband and, if possible, parents and siblings. For DNA samples, 2 parents, 1 parent, no parent, and 1 sibling were available in 10 families, 4 families, 9 families, and 1 family, respectively. None of parents of 23 index patients complained of hearing loss by clinical interview. For the normal-hearing control, 189 subjects who had normal hearing by pure-tone audiometry were used. This study was approved by the institutional ethics review board at the National Tokyo Medical Center. Written informed consent was obtained from all the subjects included in the study or their parents.

Genetic analysis

DNA was extracted from peripheral blood by standard procedures. Genetic analysis for mutations in *GJB2* and for A1555G and A3243G mitochondrial DNA mutations were conducted in all patients according to published methods (21, 22). Mutation screening of *OTOF* was performed by bidirectional sequencing of amplicons generated by PCR amplification of each exon (exons 1–50) and splice sites using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Primer sequences for *OTOF* are listed in Table S1, supporting information. Mutation nomenclature is based on genomic DNA sequence (GenBank accession number NG_009937.1), with the A of the translation initiation codon considered as +1. The nucleotide conservation between mammalian species was evaluated by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

To determine whether the prevalent p.R1939Q alleles are derived from a common founder, we conducted haplotype analysis. We genotyped single nucleotide polymorphisms (SNPs) with a minor allele frequency of >0.3 in the Japanese population and a microsatellite marker (D2S2350) spanning the *OTOF* locus and nearby genes on an ABI Genetic Analyzer 310 and Genescan 3.7 software (Applied Biosystems). Forty-four SNPs and the D2S2350 microsatellite marker in the vicinity of the mutation were genotyped in 11 AN patients who had p.R1939Q and in a part of their parents.

In six patients who did not have any mutations in *OTOF* and *GJB2* and three patients who were heterozygous for *OTOF* mutation without any mutations in *GJB2*, all coding exons and splice sites of *PJVK* were sequenced. Primer sequences were designed based on the reference sequence of *PJVK* (GenBank accession number NG_012186) and are listed in Table S2. Novelty of mutations and non-pathogenic variants found in the present study were examined in EVS (http://evs.gs.washington.edu/EVS/) and dbSNP

(http://www.ncbi.nlm.nih.gov/snp). The effect of an amino acid substitution was predicted using PolyPhen-2 software (http://genetics.bwh.harvard.edu/pph2/) and NNSPLICE 0.9 version (Berkley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/splice.html) for the splice sites. The effect of p.D1842N was also analysed by modelling the three-dimensional structure of otoferlin using SWISS-MODEL (http://swissmodel.expasy.org/) (23).

Clinical examination and data analysis

Audiological tests included otoscopic examination and pure-tone audiometry with a diagnostic audiometer in a soundproof room following International Standards Organization standards. On the basis of pure-tone air-conduction thresholds, the degree of hearing loss was determined by the better ear pure-tone average across the frequencies 0.5, 1, 2, and 4 kHz, and it was classified as mild (20–40 dB), moderate (41–70 dB), severe (71–95 dB), or profound (>95 dB) according to the recommendations for the description of audiological data by the Hereditary Hearing Loss Homepage (http://hereditaryhearingloss.org).

Results

Genetic findings

Of 23 patients with a diagnosis of congenital or early-onset AN, 13 (56.5%) carried two pathogenic *OTOF* alleles, and 3 patients (13.0%) carried one pathogenic allele (Fig. 1: inner circle). In summary, 70% of the patients had pathogenic *OTOF* alleles. Results of genotyping of the detected pathogenic *OTOF* alleles in 13 families carrying two pathogenic *OTOF* alleles (10 families in which two parents were examined and 3 families in which one parent was examined) were compatible with autosomal recessive inheritance in all these families. *OTOF* mutations consisted of three missense mutations, one frameshift mutation, two nonsense mutations, one non-stop mutation, and two putative splice site mutations (Tables 1 and 2). p.R1939Q was previously reported as a mutation and IVS47-2A>G was previously reported in dbSNP. Other seven *OTOF* mutations were novel. p.R1939Q was found in 43.5% of all alleles. We also identified 16 non-pathogenic *OTOF* variants, of which only p.P1697P was novel (Table 1). This variant did not change the score of splice site prediction. The location of each mutation in *OTOF* and the evolutionary conservation of the amino acids or nucleotides affected by the missense and putative splice site mutations are shown in Fig. 2a,b. The frequency of different *OTOF* genotypes is summarized in Fig. 1 (middle circle); 56.5% of the patients had p.R1939Q. In contrast, mutations other than p.R1939Q were confined to individual families. Previously, p.R1939Q was reported in one family with AN in the United States (19), and a different mutation in the same codon (p.R1939W) was reported in another family (16). Screening for the mutation

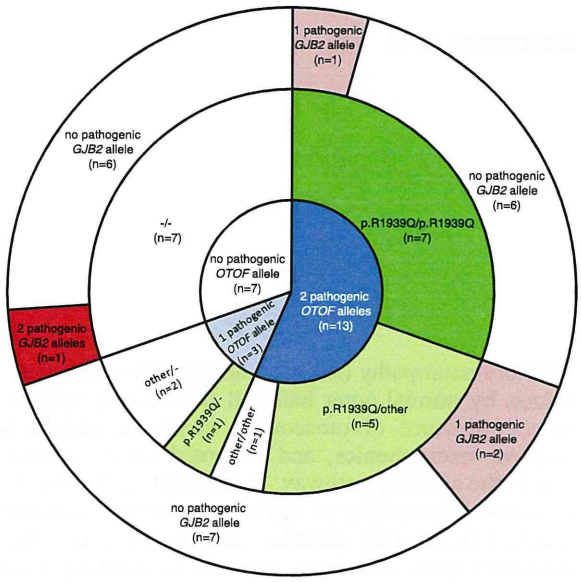

Fig. 1. Genetic backgrounds and frequency of different *OTOF* alleles in patients with congenital or early-onset auditory neuropathy (AN). (a) Distribution of patients carrying different pathogenic *OTOF* alleles (inner circle), *OTOF* genotypes (middle circle), and *GJB2* genotypes (outer circle). p.R1939Q indicates *OTOF* allele with p.R1939Q mutation; other indicates pathogenic *OTOF* alleles except for p.R1939Q allele; *n* indicates number of patients.

in 189 control subjects with normal hearing revealed only one heterozygous carrier. This mutation was predicted to be probably damaging variant according to PolyPhen-2.

The novel missense mutation p.D1842N was identified in a heterozygote without accompanying pathogenic alleles (patient 15). The mutation was predicted to be probably damaging variant according to PolyPhen-2. D1842 is located within the C2F domain, which is one of six calcium-binding modules (C2 domains) in otoferlin that are indispensable for otoferlin function. The predicted three-dimensional protein structure suggested that this mutation generates a repulsive force on calcium ions, resulting in reduced otoferlin activity (Fig. 3a–c). Another novel missense mutation, p.G541S, was identified as homozygous in a patient with parental consanguinity (patient 13). This mutation was predicted to be probably damaging variant according to PolyPhen-2 and involves a change from a non-polar residue to a polar residue in the C2C domain.

The c.1946-1965 del20 frameshift mutation truncates otoferlin at S648, causing a change in stop codon that adds six residues to the C terminus. Two nonsense mutations, p.Y474X and p.Y1822X, also truncate otoferlin. It is possible that these mutations trigger the nonsense-mediated decay response, by which aberrant mRNA is eliminated before translation (24). Even if the truncated proteins were produced, they would not function well because the mutations in c.1946-1965 del20, p.Y474X, and p.Y1822X disrupt three

Table 1. Mutations and non-pathogenic variants in congenital or early-onset auditory neuropathy

Types of variants	Location	Nucleotide variation	Predicted amino acid change	Allele frequency in normal controls	Novel or known
Mutations					
Missense substitution	Exon 15	c.1621G>A	p.G541S	0/376	Novel
	Exon 46	c.5524G>A	p.D1842N	0/376	Novel
	Exon 50	c.5816G>A	p.R1939Q	1/378	Known
Frameshift	Exon 17	c.1946-1965del20	p.R649PfsX5	0/192	Novel
Nonsense	Exon 14	c.1422T>A	p.Y474X	0/192	Novel
	Exon 46	c.5466C>G	p.Y1822X	0/192	Novel
Non-stop substitution	Exon 50	c.5992T>C	p.X1988RextX30	0/192	Novel
Putative splice site mutations					
	Exon 9 IVS	IVS9+5G>A		0/362	Novel
	Exon 47 IVS	IVS47-2A>G		0/190	Known
Non-pathogenic variants					
	Exon 2	c.129C>T	p.D43D	NT	Known
	Exon 3	c.145C>T	p.R49W	3/192	Known
	Exon 3	c.158C>T	p.A53V	75/192	Known
	Exon 4	c.244C>T	p.R82C	29/192	Known
	Exon 5	c.372A>G	p.T124T	NT	Known
	Exon 19	c.62C>T	p.P21L	172/172	Known
	Exon 23	c.2452C>T	p.R818W	0/188	Known
	Exon 24	c.2580C>G	p.V860V	NT	Known
	Exon 24	c.2613C>T	p.L861L	NT	Known
	Exon 25	c.2703G>A	p.S901S	NT	Known
	Exon 25	c.2736G>C	p.L912L	NT	Known
	Exon 41	c.4677G>A	p.V1559V	NT	Known
	Exon 41	c.4767C>T	p.R1589R	NT	Known
	Exon 43	c.5026C>T	p.R1676C	8/188	Known
	Exon 43	c.5091G>A	p.P1697P	NT	Novel
	Exon 45	c.5331C>T	p.D1777D	NT	Known

C2 domains, four C2 domains, and one C2 domain, respectively.

p.X1988RextX30, in which the stop codon is affected and 30 residues are added to the C terminus, accompanied p.R1939Q in a compound heterozygote (patient 12). Because the stop codon is separated by only one residue from the transmembrane domain, the additional C-terminal tail residues would interfere with anchoring to the membrane, which is critical for proper function. The three subjects with only one pathogenic *OTOF* allele (patient 14, patient 15, and patient 16) are likely to have mutations which could not be identified in the present study rather than just be coincidental carriers. Mutations which were not excluded in the present study include those in introns, a previously unknown exon, or a distant enhancer/promotor region as well as large deletions or other sequence rearrangements.

Screening of other genes revealed that one patient who did not have any mutations in *OTOF* was a compound heterozygote of *GJB2* mutations (patient 21). The AN phenotype has been reported in subjects with *GJB2* mutations (25). We identified three other patients with biallelic *OTOF* mutations that had heterozygous *GJB2* mutations, but they were considered to be coincidental. Distribution of patients carrying different pathogenic GJB2 alleles was shown in Fig. 1 (outer circle). None of the patients had A1555G or A3243G mitochondrial DNA mutations. Mutations in *PJVK* were not detected in six patients who did not have any mutations in *OTOF* or *GJB2* as well as in three patients who were heterozygous for *OTOF* mutation without mutations in *GJB2*.

All but one patient had a single haplotype associated with the p.R1939Q variant, which was not represented in 22 wild-type alleles in the parents, and representative SNPs and their allele frequencies as well as haplotypes are shown in Fig. 4a,b. Patient 2 had recombination of the same p.R1939Q-associated haplotype with the wild-type haplotype from his father. These results indicated that all the chromosomes carrying p.R1939Q were derived from a common ancestor.

Clinical findings

Clinical features of the patients are shown in Table 2. A consistent phenotype was present in seven patients carrying homozygous p.R1939Q and four patients who had heterozygous p.R1939Q accompanied by heterozygous truncating or putative splice site mutations. Patient 12, a compound heterozygote of p.R1939Q and a non-truncating mutation, showed a distinct phenoytpe. Patient 13, a homozygote of another non-truncating mutation, presented with temperature-sensitive AN.

Table 2. Genetic and clinical features of patients with congenital or early-onset auditory neuropathy

OTOF genotype[a]	Patient ID	Age, sex	*GJB2* genotype[a]	Degree of hearing loss (age of test)	Phenotype
p.R193Q/p.R1939Q					
	1	3, M	-/-	Profound (1 year 7 months)	NP, flat
	2	2, M	-/-	Profound (2 years 7 months)	NP, flat
	3	3, M	-/-	Profound (3 years 2 months)	NP, flat
	4	4, M	c.235delC/-	Profound (3 years 2 months)	NP, gently sloping
	5	2, F	-/-	profound (2 years 6 months)	NP, gently sloping
	6	2, M	-/-	Severe (2 years 10 months)	NP, flat
	7	2, M	-/-	Severe (1 year 9 months)	NP, flat
p.R1939Q/truncating or putative splice site[b]					
p.R1939Q/c.1946-1965del20	8	9, M	-/-	Unstable (2 years 10 months)	unstable, gently sloping
p.R1939Q/p.Y474X	9	2, M	-/-	Profound (1 year 7 months)	NP, flat
p.R1939Q/p.Y1822X	10	1, F	p.G45E+p.Y136X/-	Profound (2 years 0 month)	NP, flat
p.R1939Q/IVS9+5G>A	11	7, F	-/-	Profound (7 years 6 months)	NP, flat
p.R1939Q/non-truncating[c]					
p.R1939Q/p.X1988RextX30	12	29, F	p.V371/-	Moderate (29 years 1 month)	P, R: steeply sloping L: gently sloping
Non-truncating/non-truncating					
p.G541S/p.G541S	13	26, M	-/-	Mild[d] (25 years 11 months)	NP, flat
Various heterozygotes[e]					
p.R1939Q/-	14	5, F	-/-	Profound (5 years 10 months)	NP, flat
p.D1842N/-	15	2, F	-/-	Moderate (2 years 9 months)	NP, flat
IVS47-2A>G/-	16	6, F	-/-	Profound (5 years 11 months)	NP, flat
No mutations					
	17	4, F	-/-	Severe (4 years 8 months)	NP, gently sloping
	18	7, M	-/-	Profound (7 years 4 months)	NP, gently sloping
	19	6, F	-/-	Severe (5 years 7 months)	NP, R: gently sloping L: flat
	20	8, F	-/-	Profound (8 years 2 months)	NP, gently sloping
	21	3, F	p.235delC/c.176-191del16	Profound (3 years 1 month)	NP, flat
	22	7, F	-/-	Severe (7 years 10 months)	NP, flat
	23	2, M	-/-	Severe (1 year 8 months)	NP, flat

F, female; ID, identification number; M, male; NP, non-progressive; P, progressive; Phenotype (course of hearing loss and audiogram shape).
[a]No mutations.
[b]Truncating or putative splice site mutations.
[c]Non-truncating mutations.
[d]Temperature-sensitive auditory neuropathy.
[e]Mutations in heterozygotes without accompanying pathogenic mutations.

Patient 13 complained of difficulty in understanding conversation, and his hearing deteriorated when he became febrile or was exposed to loud noise according to his self-report. He explained that the deterioration varied from mild to complete loss of communication. Pure-tone audiometry when he was afebrile revealed mild hearing loss with a flat configuration. Among three patients who had only one pathogenic allele of *OTOF*, patient 15 carrying p.D1842N presented with moderate hearing loss, whereas patient 14 carrying p.R1939Q and patient 16 carrying IVS47-2A>G presented with profound hearing loss.

Discussion

The present study demonstrated biallelic *OTOF* mutations in 56.5% (13 of 23) of subjects with congenital or early-onset AN in Japanese population, indicating the most frequent cause associated with this type of AN. So far, biallelic *OTOF* mutations were identified in 22.2% (2 of 9) and 55% (11 of 20) of subjects with AN in American and Spanish studies, respectively (11, 12). In Brazilian population, 27.3% (3 of 11) of subjects with AN had *OTOF* mutations in two alleles (13). Taiwanese and Chinese subjects demonstrated that 18.2% (4 of 22)

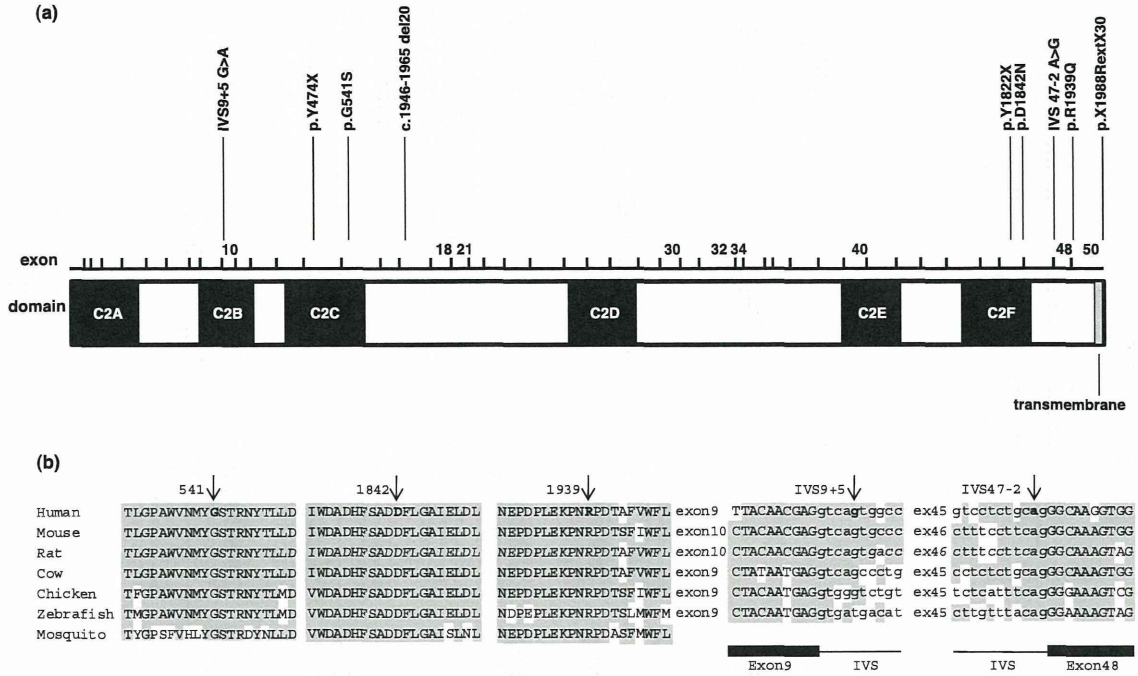

Fig. 2. The location of each mutation in *OTOF* and the evolutionary conservation of the amino acids or nucleotides affected by the missense and splice site mutations. (a) Location of mutations in the *OTOF* coding region of the cochlear isoform. Calcium-binding domains C2A through C2F are shown in black. (b) Multiple alignments of otoferlin orthologs at five non-contiguous regions and splice sites. Arrows indicate affected amino acids or nucleotides. Regions of amino acid and nucleotide sequence identity are shaded. Boundaries between introns and exons are indicated in the bottom. IVS indicates intervening sequence.

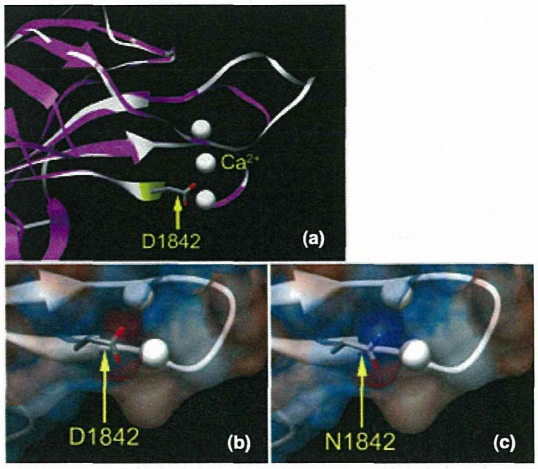

Fig. 3. Predicted three-dimensional protein structures of C2F domain in wild-type otoferlin and D1842N mutant otoferlin. (a) Ribbon model of the otoferlin C2F domain (white) superimposed onto that of the corresponding region of human protein kinase C gamma (hPKCγ, PDBID: 2UZP, chain A) which was selected as an optimal template (29.5% amino acid sequence identity) (magenta). Ca^{2+} is shown as a white sphere. The regions around D1842 of wild-type otoferlin (b) and N1842 of mutant otoferlin (c) are overlaid with their electrostatic surface potentials indicated by red (negative), blue (positive), and white (neutral). The side chains of both D1842 and N1842 are located very close (within 1.0 Å) to calcium ions. D1842N changes the electrostatic surface potential around the side chain from negative to positive in the cellular environment (pH = 7.4), and generate repulsive force on calcium ions.

and 1.4% (1 of 73), respectively, had biallelic *OTOF* mutations (14, 15).

The spectrum of *OTOF* mutations we identified differed significantly from those in other populations. Most reported *OTOF* mutations in the literature have been confined to individual families. An exception is p.Q829X, found in approximately 3% of autosomal recessive non-syndromic sensorineural hearing loss cases in the Spanish population (18). Recently, c.2905-2923delinsCTCCGAGCGCA and p.E1700Q were identified in four Argentinean families and four Taiwanese families, respectively (12, 14). In this study, p.R1939Q was detected in 13 families. Thus, p.R1939Q is now the second-most prevalent *OTOF* mutation reported. This mutation may be more common in Japan, as this mutation is found in only 1 of 10753 choromosomes in the European-American and African-American population by EVS. p.R1939Q was previously reported in one family in the United States, but the origin of the family was not detailed (19). Because no patients carrying p.R1939Q have been reported in Asian population except for the present study or in European population, this prevalent founder mutation appears to be an independent mutational event in Japanese.

Pathogenic *OTOF* mutations have been associated with stable, severe-to-profound sensorineural hearing loss with a few exceptions: c.2093+1G>T and p.P1987R were associated with stable, moderate-to-severe hearing loss (11, 19), p.E1700Q was associated with progressive, moderate-to-profound hearing

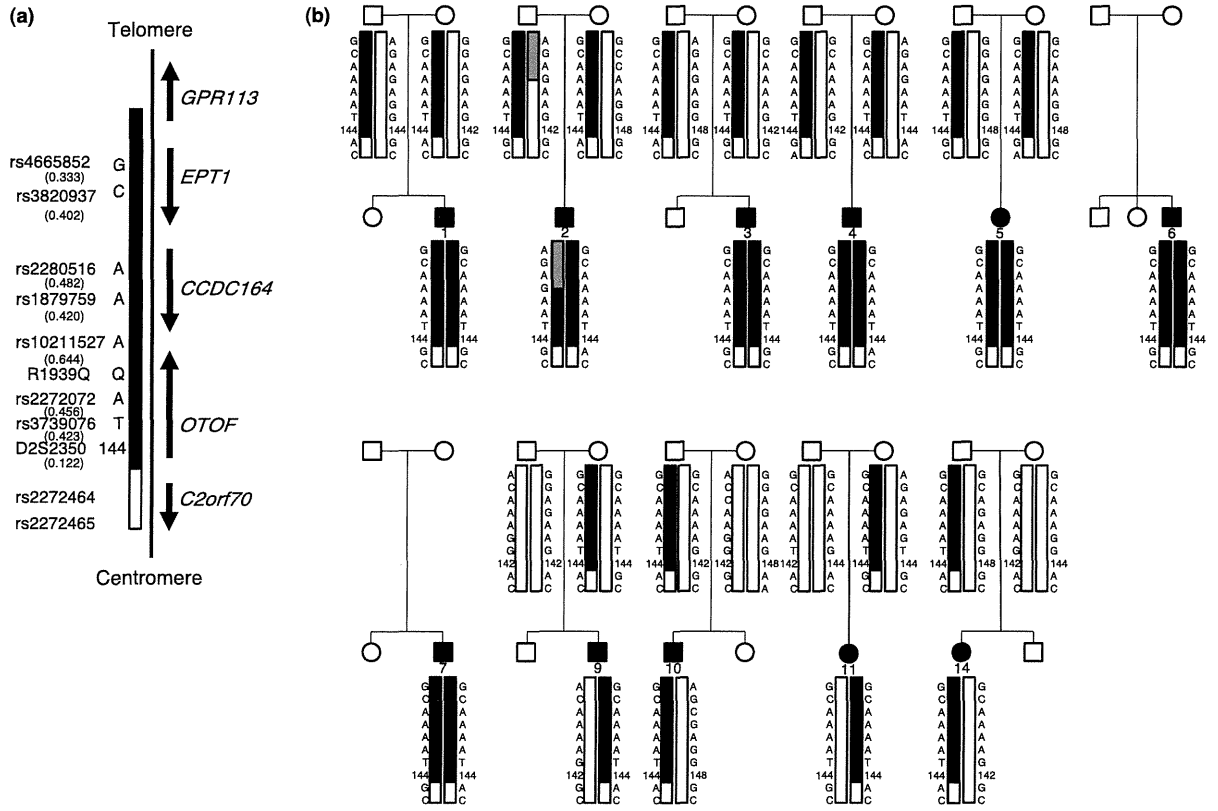

Fig. 4. Results of haplotype analysis of patients who had p.R1939Q alleles and their parents. (a) A part of tested single nucleotide polymorphisms (SNPs) and a microsatellite marker in relation to the genetic map around *OTOF* (chromosome 2p23.1). The region corresponding to the haplotype associated with the p.R1939Q mutation is indicated by black. Allele frequency of each SNP and a microsatellite marker is shown in a parenthesis. (b) Haplotypes of 11 auditory neuropathy (AN) patients with hearing loss who had p.R1939Q and their parents. The haplotype is indicated beside the vertical bars. The number under the symbol is the patient identification number in Table 2. A recombination point is indicated by grey in patient 2.

loss (14), and several mutations were associated with temperature-sensitive AN (11, 13, 15, 20). p.R1939Q homozygotes had a consistent phenotype of congenital or early-onset, stable, and severe-to-profound hearing loss with a flat or gently sloping audiogram. The same phenotype has also been reported in a family in United States, which included compound heterozygotes having p.R1939Q and a truncating mutation (19). Patients that were compound heterozygotes of p.R1939Q and truncating mutations or a putative splice site mutation also exhibited the similar phenotype in the present study. Thus, p.R1939Q variants are likely to cause severe impairment of otoferlin function. In contrast, a subject that was a compound heterozygote of p.R1939Q and a non-truncating mutation presented with a distinct phenotype of congenital or early-onset, progressive, moderate hearing loss with a steeply sloping or gently sloping audiogram. A homozygote of another non-truncating mutation also showed a distinct phenotype of temperature-sensitive AN. One of three patients who had only one allele of non-truncating mutation other than p.R1939Q presented with moderate hearing loss, whereas the other two subjects who had only one allele of p.R1939Q or a putative splice site mutation presented with profound hearing loss. These genotype–phenotype correlations of *OTOF* were similar to those of *GJB2*, i.e., more severe hearing loss was observed in subjects homozygous for truncating mutations than in subjects homozygous for non-truncating mutations, and more severe hearing loss was observed in subjects homozygous for a frameshift mutation (35delG) than in subjects compound heterozygous for the 35delG and other mutations (26, 27).

A patient with temperature-sensitive AN with a specific *OTOF* mutation was found in the present study. So far, temperature-sensitive AN has been observed in two siblings with heterozygous p.I515T without an accompanying pathogenic allele (11), three siblings with homozygous p.E1804del (20), a compound heterozygote with c.2975-2978delAG and p.R1607W (15), and a compound heterozygote with p.G614E and p.R1080P (13). The patient in this study had biallelic mutations affecting residues specific to the long isoform. Previously, two subjects showed biallelic mutations affecting this region, but they were not tested for OAE (16, 17). Thus, the present patient is the first case with biallelic mutations in this region, which indicates that mutations in the *OTOF* long isoform alone are able to cause AN.

Supporting Information

The following Supporting information is available for this article:
Table S1. Primer sequences for *OTOF*.
Table S2. Primer sequences for *PJVK*.

Additional Supporting information may be found in the online version of this article.

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High prevalence of inner-ear and/or internal auditory canal malformations in children with unilateral sensorineural hearing loss

Sawako Masuda [a,*], Satoko Usui [a], Tatsuo Matsunaga [b]

[a] Department of Otorhinolaryngology, Institute for Clinical Research, National Mie Hospital, Tsu, Mie, Japan
[b] Department of Otolaryngology, Laboratory of Auditory Disorders, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan

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ABSTRACT

Objective: Radiological and genetic examination has recently advanced for diagnosis of congenital hearing loss. The aim of this study was to elucidate the prevalence of inner-ear and/or internal auditory canal malformations in children with unilateral sensorineural hearing loss (USNHL) for better management of hearing loss and genetic and lifestyle counseling.
Methods: We conducted a retrospective study of charts and temporal bone computed tomography (CT) findings of 69 consecutive patients 0–15 years old with USNHL. In two cases, genetic examination was conducted.
Results: Of these patients, 66.7% had inner-ear and/or internal auditory canal malformations. The prevalence of malformations in infants (age <1 year) was 84.6%, which was significantly higher than that in children 1–15 years old (55.8%; $p < 0.01$). Almost half of the patients (32; 46.4%) had cochlear nerve canal stenosis; 13 of them had cochlear nerve canal stenosis alone, and in 19 it accompanied other malformations. Internal auditory canal malformations were observed in 22 subjects (31.8%), 14 (20.3%) had cochlear malformations, and 5 (7.2%) had vestibular/semicircular canal malformations. These anomalies were seen only in the affected ear, except in two of five patients with vestibular and/or semicircular canal malformations. Two patients (2.9%) had bilateral enlarged vestibular aqueducts. Mutations were found in *SLC26A4* in one of the two patients with bilateral large vestibular aqueducts. The prevalence of a narrow internal auditory canal was significantly higher in subjects with cochlear nerve canal stenosis (50.0%) than in subjects with normal cochlear nerve canals (11.1%; $p < 0.01$). There were no correlations between the type and number of malformations and hearing level.
Conclusions: The prevalence of inner-ear and/or internal auditory canal malformations detected by high-resolution temporal bone CT in children with USNHL was very high. Radiological and genetic examination provided important information to consider the pathogenesis and management of hearing loss. Temporal bone CT should be recommended to children with USNHL early in life. *SLC26A4* mutation also should be examined in cases with bilateral enlarged vestibular aqueduct.

1. Introduction

Abnormalities of the temporal bone have been associated with congenital sensorineural hearing loss (SNHL) since reported by Mondini in 1791 [1]. However, most cases of congenital SNHL were believed to be caused by abnormalities of the membranous labyrinth that could not be detected by conventional imaging techniques [2,3]. Conventional computed tomography (CT) could identify congenital cochlear malformations such as complete labyrinthine aplasia (Michel deformity), a common cavity, cochlear aplasia/hypoplasia, and incomplete partition [2–4]. Because of improvements in high-resolution CT techniques, previously unrecognized bony abnormalities—including a large vestibular aqueduct, wide and stenotic internal auditory canal (IAC), and cochlear nerve canal (CNC) stenosis—have been reported [3,5]. Currently, abnormalities found by imaging techniques not only provide diagnostic information but also aid in genetic and lifestyle counseling [1] and guide clinicians to better management of hearing loss [6].

The aim of this study was to elucidate the prevalence of inner-ear and/or IAC malformations in children with unilateral SNHL (USNHL).

* Corresponding author at: Department of Otorhinolaryngology, National Mie Hospital, 357 Osato-Kubota, Tsu, Mie 514-0125, Japan. Tel.: +81 59 232 2531; fax: +81 59 232 5994.
E-mail address: masudas@mie-m.hosp.go.jp (S. Masuda).

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2. Patients and methods

We conducted a retrospective study of charts and temporal bone CT findings of consecutive USNHL patients 0–15 years old who were seen in the Department of Otorhinolaryngology of National Mie Hospital between January 2008 and December 2011. All procedures were approved by the Ethics Review Committee of National Mie Hospital.

2.1. Subjects

The study included 69 patients. USNHL was defined as a hearing threshold greater than 30 dB hearing level for at least one frequency (500–2000 Hz). Of the 69 patients, 32 were male and 37 were female. Their ages of diagnosis ranged from 0 to 15 years (mean ± 1 SD: 4.3 ± 6.7 years, median: 4 years). The distribution of age was shown in Fig. S1. Twenty-six (37.3% of the subjects) were infants less than 1 year old. Twenty-two children had failed newborn hearing screening (NBHL) in unilateral ear and 21 of them identified USNHL in 1 year of age. One boy who had failed NBHL first visited ENT clinic and diagnosed USNHL at the age of 3 years. There was neither subjects who passed NBHL nor ones who suspected progressive hearing loss before their diagnosis. One subject had Down's syndrome and one had tetralogy of Fallot. Patients with middle ear diseases and abnormalities, conductive and combined hearing loss revealed by pure-tone audiometry, and obvious acquired hearing loss were excluded from the study.

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2.2. Audiometric evaluations

Severity of hearing loss was defined from the pure-tone average as follows: hearing level of 21–40 dB, mild; 41–70 dB, moderate; 71–95 dB, severe; and greater than 95 dB, profound [7]. Pure-tone average was defined as the average hearing threshold at 500, 1000, and 2000 Hz. Thirty-four patients in this study were too young to be examined with pure-tone audiometry initially; for these patients, USNHL was determined on the basis of auditory brainstem response (ABR) and auditory steady state response (ASSR) using an Audera® system (Grason-Stadler). Distortion product otoacoustic emissions (DPOAE) and tympanometry were performed for all subjects.

2.3. Evaluation of temporal bone CT findings

All the patients underwent high-resolution CT of temporal bone using a single-slice helical CT (HiSpeed DX/i, GE Healthcare Japan Ltd., Tokyo Hino, Japan). Contiguous 1 mm-thick sections parallel to the infraorbitomeatal line were acquired through the temporal bone, with a field of view of 230 mm, matrix size of 512 × 512, in-plane pixel size of 0.45 mm × 0.45 mm, tube voltage of 120 kV, tube current of 150 mAs and a reconstruction kernel for bone.

CT results for each patient were examined by two otologists who did not know which ear had hearing loss. Classification of inner-ear and IAC malformations was based on Sennaroglu's classification [4] and modified as follows:

1. Cochlear malformations: Michel deformity, cochlear aplasia, common cavity deformity, cochlear hypoplasia, incomplete partition type I (IP-I), incomplete partition type II (IP-II: Mondini deformity).
2. Vestibular/semicircular canal malformations: absent vestibule, hypoplastic vestibule, dilated vestibule/absent semicircular canal, hypoplastic semicircular canal, enlarged semicircular canal.
3. IAC malformations: absent, narrow, enlarged.
4. Vestibular aqueduct finding: large.
5. CNC finding: stenosis.

We defined IAC as narrow when the diameter at the level of the porous of the IAC was less than 3 mm or 2 mm smaller than the normal side and as wide when greater than 10 mm. A large vestibular aqueduct was defined as being greater than 1.5 mm at the midpoint of the vestibular aqueduct on axial images [8]. The width of the CNC was measured at its midportion. The measurements were manually obtained using calipers [5]. CNC stenosis was defined as when the width was less than 1.5 mm [9]. An example of CNC stenosis in the right ear is shown in Fig. 1.

2.4. Genetic examinations

Patients with large vestibular aqueducts participated in genetic examination. Blood samples were obtained from the proband and his/her parents. DNA was extracted from blood samples using the Gentra Puregene DNA isolation kit (Qiagen, Hamburg, Germany), and primers specific for *SLC26A4* (GenBank NG_008489) were designed. Primer sequences for *SLC26A4* are listed in Table S1, supporting information. Screening for *SLC26A4* mutations was performed by bidirectional sequencing of amplicons generated by PCR amplification of each exon (exons 1–21) and splice sites using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by SeqScape v2.6 (Applied Biosystems). Examinations were conducted only after written informed consent had been obtained from each individual or parents of the patients.

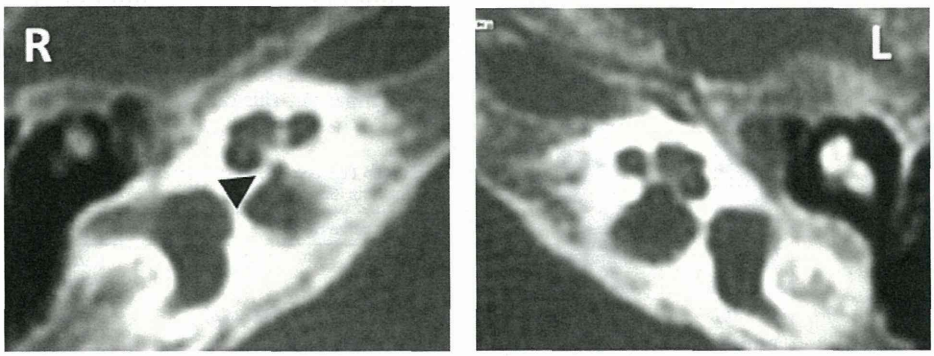

Fig. 1. Cochlear nerve canal stenosis demonstrated by transverse, thin-section CT scan of the temporal bone in three-month old boy. The left panel shows the hearing-impaired right ear (R), and the right panel shows the normal left ear (L). The arrowhead indicates the stenotic cochlear nerve canal in the right ear.

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2.5. Statistical analysis

The significance of the prevalence of the inner-ear and/or IAC malformations between infants younger than 1 year of age and children from 1 to 15 years of age, and the association between the existence of malformations and hearing level was determined by the χ^2 test.

3. Results

The prevalence of inner-ear and/or IAC malformations is shown in Fig. 2. Of the 69 subjects, 66.7% had malformations. The prevalence of malformations in infants younger than 1 year of age (84.6%) was significantly higher than that in children 1–15 years of age (55.8%; $p < 0.01$).

Table 1 shows the prevalence of each malformation. The most common anomaly was CNC stenosis of the affected ear, seen in 46.4% of the subjects. Next in frequency were IAC malformations, followed by cochlear malformations and vestibular and/or semicircular canal malformations. These anomalies were seen in the affected ear alone, except for two of five patients with vestibular and/or semicircular canal malformations. Two patients had bilateral enlarged vestibular aqueducts.

The combination of malformations we observed is summarized in Table 2. Of the 69 patients, 13 (18.8%) had CNC stenosis alone, 19 (27.5%) had CNC stenosis accompanied with other malformations, and 4 (5.8%) had narrow IAC alone. Two patients with bilateral enlarged vestibular aqueducts had cochlear or cochlear and vestibular/semicircular canal malformations. In both cases, unilateral hearing loss was found by newborn hearing screening. In one case, a 4-month-old boy, genetic examination identified a compound heterozygous mutation [p.T410M (c.1229C>T)/p.L743X (c.2228T>A)] in *SLC26A4* (Fig. S2). p.T410M was previously reported as a missense mutation [10] and p.L743X was previously reported as a nonsense mutation [11]. This nonsense substitution truncates the protein at codon 743, which is 38 amino acids from the end of the protein. This case was confirmed as Pendred syndrome. The hearing loss in his normal hearing ear developed at 1 year of age. In another case, a 2-month-old girl, pathological mutations were not found in *SLC26A4*. Her hearing level has been stable for 3 years.

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Table 3 shows the relationship between CNC malformations and IAC malformations. Of 32 cases of CNC stenosis, 16 (50.0%) were comorbid with narrow IAC. In 36 subjects with normal CNC, 4 (11.1%) had narrow IAC. The prevalence of narrow IAC was significantly higher in subjects with CNC stenosis than in subjects with normal CNC ($p < 0.01$).

Table 4 shows the combination of malformations and hearing level. There were 6 cases of mild hearing loss, 13 cases of moderate hearing loss, 7 cases of severe hearing loss, and 43 cases of profound hearing loss. DPOAE was absent in the affected ear in all subjects, except for two patients with unilateral profound hearing loss with CNC stenosis and narrow IAC without cochlear/vestibular/semicircular canal malformations. These two patients demonstrated normal responses in DPOAE in both ears. In one of these cases, ABR was performed. The threshold of wave V was 95 dBnHL (normal Hearing Level) in the affected ear and 20 dBnHL in the normal ear. This case was confirmed as unilateral auditory

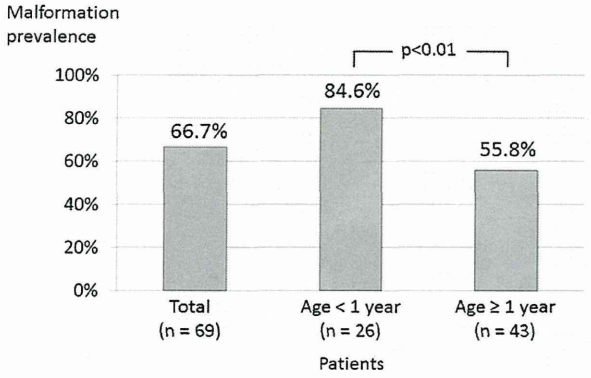

Fig. 2. Prevalence of inner-ear and/or internal auditory canal malformations found by temporal bone computed tomography.

Table 1
Prevalence of each malformation.

Malformation	Number (prevalence)
Cochlea	14 (20.3%)
Cochlear aplasia	0
Common cavity deformity	2 (2.9%)
Cochlear hypoplasia	1 (1.4%)
Incomplete partition (IP-I, IP-II)	11 (15.9%)
Vestibular/semicircular canal	5[a] (7.2%)
Internal auditory canal	22 (31.8%)
Narrow	20 (29.0%)
Enlarged	1 (1.4%)
Absent	1 (1.4%)
Vestibular aqueduct: enlarged (bilateral)	2 (2.9%)
Cochlear nerve canal: stenosis	32 (46.4%)

[a] Two cases had malformation in both ears.

Table 2
Combination of malformations.

Combination of malformations	Number (percentage)
CNC stenosis	13 (18.8)
CNC stenosis + narrow IAC	10 (14.5)
CNC stenosis + narrow IAC + C malformations	5 (7.2)
CNC stenosis + narrow IAC + V/SC malformations	1[a] (1.4)
CNC stenosis + C malformations	3 (4.3)
Narrow IAC	4 (5.8)
Large IAC	1 (1.4)
C malformations	2 (2.9)
C/V/SC malformations	2 (2.9)
V/SC malformations	2[b] (2.9)
Large VA + C malformations	1 (1.4)
Large VA + C malformations + V/SC malformations	1 (1.4)
CC with absent IAC	1 (1.4)
Normal	23 (33.3)
Total	69 (100.0)

CNC stenosis, cochlear nerve canal stenosis; IAC, internal auditory canal; C, Cochlear; V/SC, vestibular/semicircular canal; VA, vestibular aqueduct; CC: common cavity.
[a] This patient had bilateral V/SC malformations.
[b] One patient had bilateral V/SC malformations.

Table 3
Relationship between cochlear nerve canal malformations and internal auditory canal malformations.

Cochlear nerve canal		Internal auditory canal	
Stenosis	32 (46.4%)	Narrow	16 (50.0%)
		Normal	16 (50.0%)
Normal	36 (52.2%)	Narrow	4 (11.1%)
		Normal	31 (86.1%)
		Large	1 (2.8%)
Absent	1 (1.4%)	Absent	1 (100.0%)

Table 4
Combination of malformations and hearing level.

Cochlear nerve canal stenosis		+				–				Total
Narrow internal auditory canal		+		–		+		–		
Cochlear/vestibular/semicircular canal malformations		+	–	+	–	+	–	+	–	
Hearing level	Mild (21–40 dB)			1			1		4	6
	Moderate (41–70 dB)	1			3		1	4	4	13
	Severe (71–95 dB)	2			1			1	3	7
	Profound (>95 dB)	4[a]	10[b]	2	9		2	4	12	43
Total		7	10	3	13		4	9	23	69

[a] One patient had common cavity with IAC deficiency.
[b] Two patients demonstrated normal distortion product otoacoustic emissions.

neuropathy spectrum disorder. There were no correlations between the hearing level and the existence of CNC stenosis, narrow IAC, or other malformations in subjects with absence of DPOAE.

4. Discussion

The data in the present study showed a high prevalence of inner-ear and/or IAC malformations in pediatric USNHL. The prevalence was 84.6% in infants younger than 1 year of age. Most USNHL in these infants was considered as congenital, implying that more than 80% of the congenital USNHL was caused by morphological abnormality accompanied with bony anomalies.

The frequency of reported abnormal temporal bone findings in patients with USNHL varies from 7% to 44% [7]. Song et al. [8] studied CT of 322 children with USNHL and reported that 28.9% had malformations. Simons et al. [7] reported that the prevalence of CT abnormalities was 35% (29 of 83 cases), and the prevalence of magnetic resonance imaging (MRI) abnormalities was 25% (10 of 40 cases) in children with USNHL. However, they did not refer to the CNC.

The size of the CNC was first reported by Fatterpekar et al. in 2000 [5]. They demonstrated that the length and width of the CNC were significantly smaller ($p < 0.05$) in patients with congenital SNHL who had "normal" findings at thin-section temporal bone CT than in the control group. In 2008, Kono [3] investigated 118 patients without inner-ear malformations among 160 patients with USNHL, and 60% showed a significant difference in the CNC diameters between the affected and unaffected sides. Kono suggested that a diameter of less than 1.7 mm on transverse images or less than 1.8 mm on coronal images was hypoplasia. Stjernholm et al. [12] suggested that if the CNC diameter was less than 1.4 mm, then the possibility of cochlear nerve abnormality should be considered. Recent studies [9,13] demonstrated that CNC stenosis with a diameter of 1.5 mm or less as assessed with CT suggested cochlear nerve deficiency or hypoplasia as assessed with MRI. Wilkins et al. [14] showed a significant correlation between the degree of CNC stenosis and the degree of hearing loss. In the present study with the definition that the diameter was less than 1.5 mm, 46.4% of the subjects had CNC stenosis.

The exact cause of narrow CNC is unclear. Proper development of the IAC requires the presence of a normal cochlear nerve as a stimulus for attaining normal adult dimensions [5]. There is a possibility that the normal development of the CNC similarly needs the nerve for stimulus [5,15]. Fatterpekar et al. [5] speculated that, in patients with abnormality involving the membranous labyrinth, inhibition of the normal trophic effects of nerve growth factors owing to a diminutive cochlear nerve results in a small CNC. That is to say, hypoplasia of the CNC might be secondary to a hypoplastic cochlear nerve associated with some abnormality of a membranous labyrinth that could not be detected by current imaging techniques [3]. Very few of our subjects demonstrated a positive response in DPOAE, suggesting that at least the outer hair cells were affected or may not exist in most patients with USNHL.

The abnormalities found by imaging techniques provide information for diagnosis, management of hearing loss, and genetic and lifestyle counseling [1,6]. Congenital malformed inner ears may be associated with cerebrospinal fluid leakage, and thus development of meningitis is a very real possibility. Parents of children with inner-ear anomalies should be informed of the early symptoms and signs of meningitis. Consideration also should be given to immunization against common organisms implicated in meningitis [16]. Genetic examination should be recommended for patients with enlarged vestibular aqueducts. Pourova et al. [17] recommend performing SLC26A4 mutation analysis, following GJB2 analysis, in all hearing loss patients with bilateral enlarged vestibular aqueduct and/or associated thyroid impairment. Thy also mentioned that it is not reasonable to test the SLC26A4 gene in children with sporadic deafness without knowledge of their temporal bone CT/MRI images or even with its normal result. Mutations in the SLC26A4 are responsible for Pendred syndrome [18] as well as DFNB4 (non-syndromic hearing loss with inner ear abnormalities—enlarged vestibular aqueduct and/or Mondini deformity) [19]. Pendred syndrome and bilateral enlarged vestibular aqueduct correlates with the presence of two mutant alleles of *SLC26A4* [17,20,21]. Hearing loss in most patients with *SLC26A4* mutations fluctuates and is progressive [22]. Mutations in *SLC26A4* indicate the necessity for careful management of hearing and comorbidities, such as goiter.

The lack of MRI examination is one of the limitations in the present study. The results suggest the importance of temporal bone CT. Nevertheless, the risks of sedation/anesthesia for imaging in infants and young children, or indeed the radiation risk should be considered. The ideal imaging algorithm in children with unilateral or asymmetric SNHL is controversial [7]. MRI can detect soft-tissue abnormalities such as cochlear nerve deficiency with normal CNC and IAC. Simons et al. [7] suggested that virtually all children with SNHL should have an imaging study as part of their workup. They prefer high-resolution temporal bone CT as the initial study because of a high prevalence of positive findings and less cumbersome logistical issues. They also recommended that a negative CT scan should be followed by MRI to rule out SNHL caused by the central nervous system.

There are some other limitations regarding the current study. The first limitation is the diagnosis of SNHL. USNHL was determined on the basis of ABR and ASSR in 34 young patients. Middle-ear diseases and abnormalities were ruled out by CT and tympanometry; however, there is a possibility that some patients had conductive or combined hearing loss. Another limitation concerns the number of subjects. We examined 69 children, however, the evaluations should be need in the larger group.

In conclusion, a high prevalence of inner-ear and/or IAC malformations was detected by high-resolution temporal bone CT in children with USNHL. Radiological and genetic examination provided important information concerning the pathogenesis and management of hearing loss. The results of this study supported the recommendation of temporal bone CT to children with USNHL early in life. Genetic examination of *SLC26A4* also should be performed in all cases with bilateral enlarged vestibular aqueduct. The study in the larger group will likely refine the clinical protocol.

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